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Estrogen metabolites in a small cohort of patients with idiopathic pulmonary arterial hypertension

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Increased risk and severity of idiopathic pulmonary arterial hypertension (iPAH) is associated with elevated plasma estradiol (E2) in both men and postmenopausal women (1, 2), suggesting that E2 is not ovarian-derived. Aromatase is responsible for the synthesis of E2. Remodelled pulmonary arteries, lesions and human pulmonary artery smooth muscle cells (hPASMCs) from PAH patients have high levels of aromatase (3). CYP1B1 can convert E2 to mitogenic 16-hydroxy-estrogens (16OHEs) and this enzyme is over-expressed in diseased pulmonary arteries from PAH patients (4). Existing assays have not been sensitive or specific enough to detect levels of many E2 metabolites in PAH patient blood. Using a newly developed liquid chromatography tandem mass spectrometry (LC-MS/MS) assay (5), here we investigate the profile of several E2 metabolites in iPAH patients.

Methods

Serum from 22 subjects with iPAH (12 males, 10 females) and 34 healthy control subjects (17 males, 17 females) were analysed by LC-MS/MS for estrone (E1), 17 α -estradiol (17 α E2), 17 β -estradiol (E2), 16-Hydroxyestrone (16OHE1), 16-Hydroxyestradiol (16OHE2), 2-methoxyestrone (2MeOE1), 4-methoxyestrone (4MeOE1), 2-methoxyestradiol (2MeOE2) and 4-methoxyestradiol (4MeOE2) (5). Data were compared by a Mann-Whitney U-test with associations assessed using Pearson's correlations. Values below the confirmed limit of quantification (LOQ) were imputed as the LOQ (2pg/mL E1, E2, 16OHE2, 2MeOE1 and 6pg/mL 16OHE1).

To determine if 16OHE2 was present at bioactive concentrations, proliferation was studied in hPASMCs and cell migration studied in human blood outgrowth endothelial cells (BOECs). hPASMCs were isolated from distal pulmonary arteries (0.3 – 1 mm diameter) from macroscopically normal controls or patients undergoing lung transplantation. BOECs were isolated from PAH patients as described previously (6) with cells cultured as described previously (7). The patients were male and female (heritable PAH/iPAH). Cells were stimulated with 16OHE2 following preliminary concentration-response analysis (10nM hPASMCs and 1nM BOECs) for 48 hours in phenol red-free media. Cell counts and cell migration (Corning® Transwell inserts) were assessed using a Countess II FL cell counter (Life Technologies, UK). A student's t-test was applied for comparison of 16OHE2 vs vehicle treated cells for each group.

Results

E1 was decreased in serum of female iPAH patients whilst 16OHE2 was increased. E1, E2 and 16OHE1 were elevated in male iPAH patients (Table 1). Comparing males and females, E1 was higher in female controls compared with male controls ($p=0.01$). In iPAH, E1 was higher in males than females ($P=0.03$).

In female iPAH patients, increased E1 was associated with increasing PAWP ($R^2=0.5$, $\beta=0.7$, $p=0.03$), decreased E2 was associated with increasing age ($R^2=0.7$, $\beta=-0.8$, $p=0.02$), and increasing E2 with increasing PAWP ($R^2=0.6$, $\beta=0.6$, $p=0.05$) while increased 16OHE1 correlated with a reduction of cardiac output ($R^2=0.7$, $\beta=-0.6$, $p=0.01$). In male iPAH, increased E2 was associated with increased PAWP ($R^2=0.5$, $\beta=0.6$, $p=0.02$) while increased E1 and E2 associated with increasing BNP levels ($R^2=0.6$, $\beta=0.8$, $p=0.01$ and $R^2=0.4$, $\beta=0.6$, $p=0.04$).

16OHE2 caused proliferation of hPASMCs from female PAH patients (by 63%) but not controls (male or female) (Figure 1A-C). 16OHE2 caused increased migration of BOECs from PAH patients, both in males (by 55%) and females (by 67%) (Figure 1D).

Discussion

This study is the first to simultaneously quantify several estrogens in controls and PAH patients as our methodological paper measured levels in pooled plasma samples (5). LC-MS/MS allowed discrimination between isomers of estradiol and its bioactive metabolites such as 16OHE1, 16OHE2 and methoxyestrogens. Increased 16-hydroxylation of E1/E2 promotes formation of proliferative metabolites and we identified accumulation of 16OHE2 in female iPAH patient serum for the first time. We confirmed increased levels of 16OHE1 in patients which had been suggested by previous studies (8, 9). In female iPAH, 16OHE2 was also increased. There was increased E1 and E2 levels in male iPAH patients, consistent with a previous study using immunoassay detection (1). The E2 concentrations we determined here were lower compared with previous studies determined using immunoassays. This is consistent with immunoassays being limited by cross-reactivity and over-estimation of E2 levels compared with LC-MS/MS (10).

Aromatase and CYP1B1 are increased in PAH patient pulmonary arteries and pulmonary vascular lesions (3, 4). Increased aromatase activity may account for increased plasma E2 in men and post-menopausal women with iPAH (1, 2). In female iPAH, there was a decrease in E1 with no change in E2. The change in E1 may reflect an increased conversion to 16OHEs via CYP enzymes as reflected by increased 16OHE1 and 16OHE2 in these patients. Where there is a decrease in E1 with no change in E2 this could also be due to the increased metabolism of E2. Baird et al. recently demonstrated that E2 levels are elevated in post-menopausal iPAH female patients compared with controls; however, E2 levels from controls in that study were 5-7 pg/mL, commensurate with post-menopause levels (2). The majority of females in this current study were pre-menopausal, with sampling not routinely timed to consider menstrual cycle, and mean E2 concentrations in controls and patients were ~34 pg/mL. Hence, it is perhaps unlikely that a further increase would be expected in this patient cohort. Future clinical studies could consider blood sampling from pre-menopausal women at the same stage of the menstrual cycle. 3

As 16OHE1 can be derived directly from E1 and male patients have higher E1, this may also contribute to the elevated 16OHE1 in these patients. Consistent with increased 16OHEs observed in iPAH patients, polymorphisms in *CYP1B1* are observed in PAH patients associated with functional differences in 16-hydroxylation, cancer risk and E2-mediated carcinogenicity (11, 12). Conversion of E2 to 16OHE1 by CYP1B1 may contribute to the pathogenesis of experimental pulmonary hypertension and cause proliferation of hPASMCs via increased oxidative stress (7, 8).

We have already demonstrated that 16OHE1 can cause proliferation of both male and female hPASMCs and male mouse PASMCs at concentrations observed in plasma (4, 7, 13). 16OHE1 acts through the ER α receptor in hPASMCs (7, 14) and indeed we have shown that ER α receptors are more highly expressed in female PAH hPASMCs (15). This is consistent with female hPASMCs being more proliferative to E2, also due to decreased basal bone morphogenetic protein receptor II (BMPRII) signalling in female hPASMCs [16]. 16OHE2 is a novel metabolite with unknown effects that was increased in the serum from female iPAH patients compared with controls. This could also be due to altered CYP1B1-mediated 16-hydroxylation (4, 13). 16OHE2 is an E2-receptor agonist, capable of cellular proliferation associated with cancer risk and E2-mediated carcinogenicity (12). Consistent with this, we now show that, at a pathophysiological concentration, 16OHE2 can also induce proliferation in female human PAH PASMCs with no effect in female or male control PASMCs where ER α expression is not so high. However, we show that 16OHE2 increases cell migration in both male and female PAH BOECs. As 16OHE1 has been shown to act as

a 'second hit' to uncover a pulmonary hypertensive phenotype in BMPRII mutant male and female mice [9], this suggests that both 16OHE1 and 16OHE2 may also facilitate pulmonary vascular remodelling in males and females by acting as a 'second hit' to BMPRII mutations. 16OHE2 is also produced in abundance during pregnancy and it is interesting to speculate that it may contribute to pregnancy being a risk factor for PAH (17).

It should be noted that all of the medications that the patients were prescribed are metabolised in the liver by CYP enzymes such as CYP3A4 and CYP2C9/8 and have no known effects on lung or liver CYP1B1 or CYP1A1 activity. One limitation of this study is that the patients were older than the controls. However E2 and 16OHE1 levels correlated with indicators of disease severity within the patient groups and decreased E2 was associated with increasing age in iPAH patients. This suggests that increased E2/metabolite levels in PAH patients are related to disease status rather than difference in age.

The results suggest that estrogen metabolism is altered in PAH and both 16OHE1 and 16OHE2 accumulate, with 16OHE1 levels relating to disease severity. This study is limited by the small size but important as it warrants further multi-center fully powered studies to confirm and correlate metabolite levels with severity and sub-groups of PAH. Future studies on CYP enzymes and CYP1B1 inhibitors in patients with PAH may also be valuable.

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Table 1: Clinical Characteristics and estrogen Concentrations for Controls vs iPAH Patients

	Female Controls	Female iPAH	P-Values	Male Controls	Male iPAH	P- Values
Subjects	17	10		17	12	
Clinical Characteristics						
Age (y)	39.0 [31 – 45]	56.5 [43 - 61]	0.01	32.5 [27 – 37]	57.0 [50 – 62]	0.001
BMI (kg·m ⁻²)	24.9 [22 – 28]	29.1 [28 – 31]	0.06	23.8 [22 – 33]	32.8 [29 – 34]	0.07
Right Atrial Pressure (mmHg)		6.0 [4 – 10]			9.0 [7 - 14]	0.23
mPAP (mmHg)		55.0 [35 - 62]			51.5 [40 - 60]	0.97
Cardiac Output (L/min)		5.3 [5 - 7]			4.6 [4 - 6]	0.38
PAWP (mmHg)		9.0 [8 - 12]			11.0 [9 - 16]	0.23
PVR (mmHg/l·min)		9.0 [4 - 13]			9.1 [4 -11]	0.97
BNP (pg/mL)		76.0 [25 - 238]			114.0 [21 - 350]	0.79
Current PAH Medications						
ERA		3			4	
PDE5 Inhibitor		2			4	
Epoprostenol or Trepstinil		2			2	
Calcium Channel Blocker		-			1	
Treatment Naïve		-			1	
Estrogen Concentrations (pg/mL)						
E1	34.9 [27 – 75] (100)	16.9 [9 – 34] (91)	0.02	23.6 [17 – 32] (100)	28.1 [24 – 55] (100)	0.05
E2	16.8 [10 – 59] (100)	7.9 [4 – 65] (100)	0.15	16.0 [10 – 21] (100)	21.3 [19 – 30] (100)	0.02
16OHE1	24.8 [11 – 38] (65)	29.8 [17 – 53] (91)	0.09	24.1 [19 – 32] (47)	79.9 [58 - 96] (75)	0.01
16OHE2	10.7 [9 – 11] (18)	14.6 [8 – 26] (82)	0.01	15.4 [8 – 18] (41)	15.9 [6 – 3] (67)	0.24
2MeOE1	8.4 [6 – 26] (41)	6.9 [6 – 80] (55)	1.00	10.9 [9 – 26] (30)	13.2 (8)	-

4MeOE1	ND	ND	-	ND	ND	-
2MeOE2	ND	ND	-	ND	ND	-
4MeOE2	ND	ND	-	ND	ND	-
<50 y Estrogen Concentrations (pg/mL)	n=14	n=4				
E1	33.7 [27 - 48]	39.5 [24 - 52]	1.00			
E2	24.0 [12 - 64]	47.7 [25 - 88]	0.57			
16OHE1	21.9 [12 - 35]	34.4 [16 - 52]	0.23			
16OHE2	10.1 [9 - 11]	14.0 [10 - 15]	0.06			
2MeOE1	8.4 [7 - 18]	7.0	0.64			
≥ 50 y Estrogen Concentrations (pg/mL)	n=3	n=6				
E1	78.6 [56 - 107]	12.1 [9 - 19]	0.02			
E2	9.6 [7 - 13]	4.6 [3 - 7]	0.26			
16OHE1	37.7	29.8 [27 - 38]	0.38			
16OHE2	16.5	21.1 [9 - 28]	0.26			
2MeOE1	16.0 [11 - 21]	6.5 [6 - 36]	0.90			

Endothelin receptor antagonist, ERA; Phosphodiesterase type 5 inhibitor, PDE5; Pulmonary arterial hypertension, PAH; idiopathic PAH, iPAH; Mean pulmonary artery pressure, mPAP; Pulmonary artery wedge pressure, PAWP; Pulmonary Vascular Resistance, PVR; B-type natriuretic peptide, BNP; Not Detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 4-Methoxyestrone, 4MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2. Medication details of 3 female patients is unknown. Clinical characteristics and estrogen Levels shown as median [Q₁ – Q₃] (% LC-MS/MS Detection). P-values following a Mann Whitney U test in control vs iPAH or female iPAH vs male iPAH clinical characteristics.

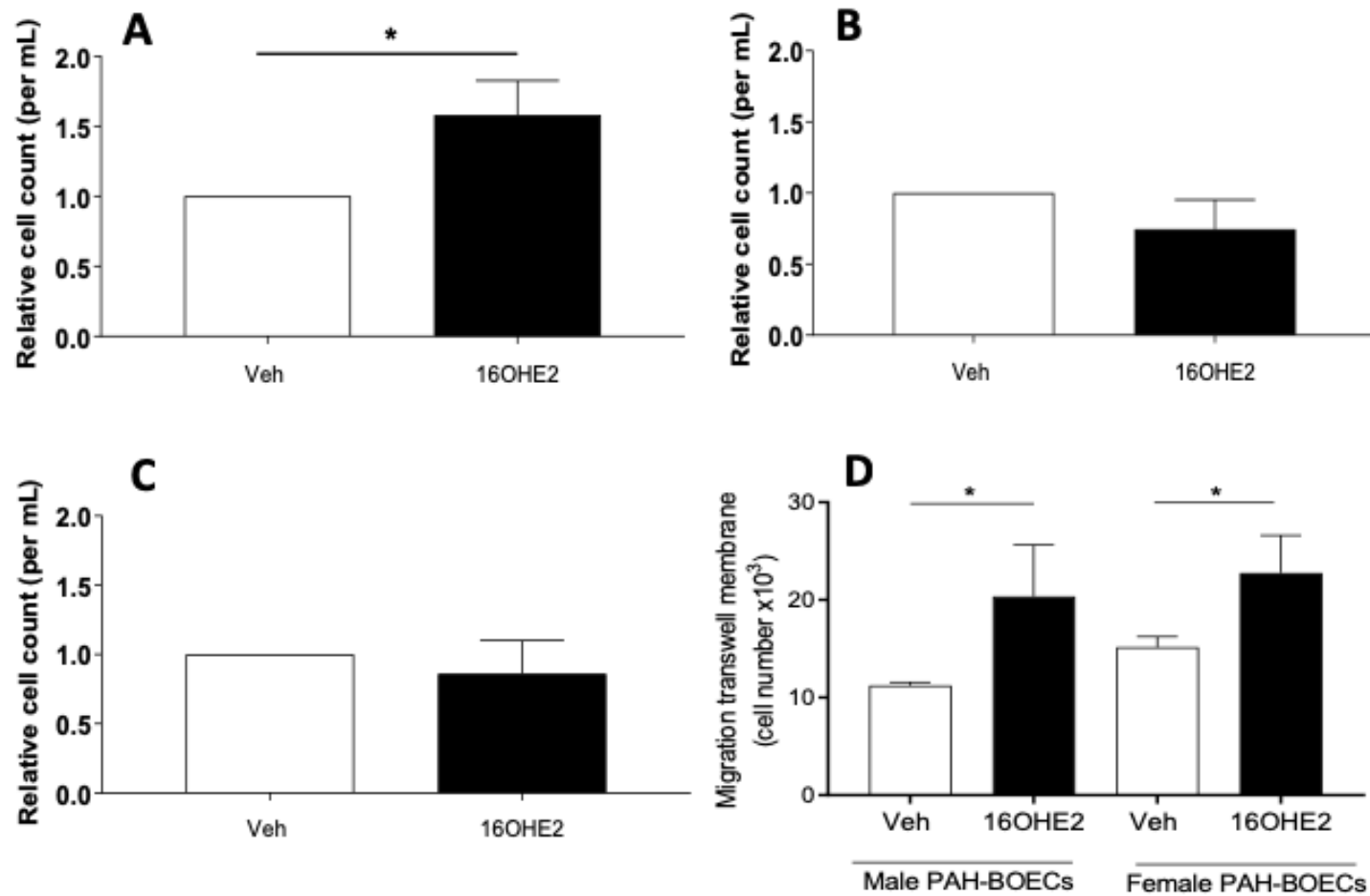


Figure 1: 16OHE2 stimulation increases proliferation and migration of human cells

10 nM 16OHE2 increases proliferation in female PAH (A) with no effect on female control (B) and male control (C) human PSMCs. 1 nM 16OHE2 increased migration of male and female BOECs derived from PAH patients (D). Data shown as Mean \pm SEM (n= 4 - 6) with statistics performed by comparison of 16OHE2 to veh by a student's t test *p<0.05, **p<0.01. Veh = Vehicle Control, Ethanol.